(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 November 2003 (20.11.2003)

PCT

(10) International Publication Number
WO 03/095496 A1

(51) International Patent Classification[7]: C08B 37/00, C08G 83/00, 65/331, C07K 2/00

(21) International Application Number: PCT/EP03/04790

(22) International Filing Date: 7 May 2003 (07.05.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
02090169.0 8 May 2002 (08.05.2002) EP

(71) Applicant *(for all designated States except US)*: **MEDICAL ENZYMES AG** [DE/DE]; Teltowkanalstr. 2, 12247 Berlin (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KRÄHMER, Ralf** [DE/DE]; Binzstr. 35, 13189 Berlin (DE). **MÜLLER, Angela** [DE/DE]; Solmsstr. 17, 10961 Berlin (DE). **LEENDERS, Frank** [DE/DE]; Meyerheimstr. 7, 10439 Berlin (DE).

(74) Agents: **RASCH, Dorit, R.** et al.; Gulde Hengelhaupt Ziebig & Schneider, Schützenstr. 15-17, 10117 Berlin (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/095496 A1

(54) Title: ACTIVE CARBOHYDRATE CONTAINING PROTECTING REAGENTS FOR CHEMICAL MODIFICATIONS, THEIR PRODUCTION AND USE

(57) Abstract: The present invention relates to new active carbohydrate containing protecting reagents represented by a carbohydrate central unit, which is attached to a polymer chain at least at one of the hydroxyl groups and to an active linkage group at the anomeric position of the carbohydrate central unit. These new compounds are active carbohydrate containing protecting reagents, glycodendrimers and related hydrophilic oligomers. Further the invention relates to a method for their preparation and their use for production of biologically active molecules, which are modified by these reagents preferred for biotechnological use.

Active carbohydrate containing protecting reagents for chemical modifications, their production and use

Description

The present invention relates to new active carbohydrate containing protecting reagents represented by a carbohydrate central unit, which is attached to a polymer chain at least at one of the hydroxyl groups and to an active linkage group at the anomeric position of the carbohydrate central unit. These new compounds are active carbohydrate containing protecting reagents, glycodendrimers and related hydrophilic oligomers. Further the invention relates to a method for their preparation and their use for production of biologically active molecules, which are modified by these reagents preferred for biotechnological use.

The new age of biotechnology is coming up with more and more biological active macromolecules isolated and produced from different organisms, respectively. Furthermore, the protein-based pharmaceuticals urge increasingly into the drug market. Many biotechnologically produced pharmaceuticals suffer from the problems of relatively short half life *in vivo* (clearance and immune-mediated inactivation) and strong immunogenicity.

One possibility to get around the above described disadvantages is the chemical modification via the covalent attachment of hydrophilic polymers to bio-active molecules or drug carriers. It is also observed that the modification of small molecule drugs with such polymers leads to a lower toxicity and shows other beneficial effects on pharmaceutical properties of the small molecule drug.

The most common hydrophilic polymer is polyethylene glycol, which is simply called "PEG". A reason for its common use is the low toxicity of the polymer PEG, which means it is bio-compatible. As an example for biotechnological application the commercial available, activated polymer PEG is attached to proteins and enzymes. Such conjugates have shown *in vivo* a dramatically increased half life, reduced toxicity and a decreased rate of kidney clearance. Furthermore, the conjugates have shown an enhanced solubility in aqueous systems. A good

overview about such applications is given by N. K. Jain et al., Pharmazie 2002, 57, 5 – 29 "Pegnology: a review of PEG-ylated systems".

In the case of small molecule drugs it has been shown, that conjugates containing the chemotherapeutic Doxorubicin show a significantly decreased toxicity in comparison with the unmodified Doxorubicin (F. Kratz et al., Bioorganic & Medicinal Chemistry 1999, 7, 2517-2524).

These days a lot of procedures exist to covalently modify proteins with bio-compatible polymers like PEG or other hydrophilic polymers. The modification of proteins aims the extension of circulation time and the avoidance of immunogenicity or toxicity, respectively (Zalipsky, S. Advanced Drug Delivery Reviews, 1995, 16, 157-182).

Even though, this technology is far developed there remain some significant disadvantages: The modification of biopharmaceuticals with PEG in many cases leads to a dramatic decrease of their biological activity. Furthermore, polymers like PEG are characterized by a broad range of different molecular weights (poly-disperse), which causes problems with the reproducibility of production and/or modification, respectively. Depending on the quality of the mPEG (monomethoxyPEG) and the type of activation in some cases unwanted cross-linking reactions occur.

Summarizing, there is a great need for new hydrophilic oligomers or polymers, which can be used for the modification of biotechnological products (e.g. proteins, surfaces) or small drugs.
The present invention is directed to overcome these deficiencies. The object on which the invention is based on is to generate such hydrophilic substances, which can be used for the modification of biotechnological products and small molecule drugs. In view of the above described state of the art, the present invention has for its object to provide polymers having activated linker groups which react with amino functional groups of proteins or peptides and other functional groups of biotechnological products in aqueous solution and under mild conditions.

The present invention provides active carbohydrate containing protecting reagents represented by a carbohydrate central unit, which is attached to a polymer chain at

least at one of its hydroxyl group and to an activated linkage group at the anomeric position *(position 1)* of the carbohydrate central unit, a *process* (method) for the synthesis of this class of compounds and their use for the covalent coupling to functional groups of biotechnological products and small molecule drugs as described below. The active carbohydrate containing protecting reagents according to the current invention has a single activated functional group (e.g. active ester), which will not support any cross linking reactions.

These new active carbohydrate containing compounds are hydrophilic, bio-compatible compounds. They are easy to prepare and the properties enable a broad field of application. Conjugates of these active carbohydrate containing compounds with biological active substances are suitable for prolonged therapies. Furthermore, the new carbohydrate containing compounds are also suitable for using them as medical material, namely for administering therapeutic amounts of conjugates of biological active compounds, which are containing the new masking reagent, for example for treatment of viral or bacterial infections or for cancer treatment.

In accordance with the invention conjugates of the active carbohydrate containing protecting reagents with biological active compounds such as proteins (e.g. human growth factors), enzymes, liposomes, antibodies, drugs, phospholipids, lipids, nucleosides, oligonucleotides, microorganisms, human cells and surfaces are also provided.

The active carbohydrate containing protecting reagents are represented by the general formula I

I,

wherein a glycoside central unit G is attached to a polymer chain at least at one of the hydroxyl groups and to an activated linkage group at the anomeric position (1-position), which is able to react readily with other functional groups
and wherein

G is a glycoside central unit, which is a monosaccharide, an oligo- and polysaccharide, respectively, consisting monosaccharide units, an amino sugar or a polyol,

R-poly- is
a polymer chain with one or more recurring monomer units or
a repeating building block **II** which is attached via the linkage group to a further glycoside unit G

P-HN-poly-X-G
ζ
Z - (CH_2)m – CO- **II,**

wherein P is an amino protecting group or the next repeating building block or a terminal unit **I**,

whereas

R is H, OH or selected from the group consisting of alkyl, O-alkyl (1 to 20 C atoms), benzyl, aryl, acetal, aldehyde, alkenyl, acrylate, acrylamide, active sulfone, alkyl amine, protected alkyl amine or amine, thiol and protected thiol, or also selected from the group consisting of a monosaccharide, an oligo- and polysaccharide, respectively consisting monosaccharide units, an amino carbohydrate or a polyol, and

poly is selected from a poly alkylene oxide, a poly oxyethylated polyol, a poly olefinic alcohol and a poly acrylomorpholine,

X is selected from --O--, --S--, –NH–, -NHCO–, –CONH-, -$NHCO_2$--, O_2CNH–, -CSNH– and --NHCS–,

m is 1 to 10, preferred 1 to 6

Y is selected from a group of (O-alkyl)$_2$, -$OSO_2CH_2CF_3$ (Tresyl), – CO – Q, maleimide, -O-CO-nitrophenyl or – trichlorophenyl, -S-alkyl (C_1 to C_6), -S-S-alkyl, -S-aryl, -S-S-aryl, -S-benzyl, -S-alkyl (C_1 to C_6)-OH, -S-alkyl(C_1 to C_6)-NH_2, -S-alkyl (C_1 to C_6)-$OSO_2CH_2CF_3$ and -S-alkyl (C_1 to C_6)-CO_2-Q-, -SO-alkenyl, -Halogen (Cl, Br, I),

wherein

Q is H or OH or is selected from a group of O-aryl, O-benzyl,

O-N-succinimide, O-N-sulfosuccinimide, O-N-phthalimide, O-N-glutarimide, O-N-tetrahydrophtalimide, -N-norbornene-2,3-dicarboximide, hydroxybenzotriazole and hydroxy-7-azabenzotriazole

and

Z is selected from -O-, -S- or –NH-, -N(alkyl C_1 to C_{20})-, -N(aryl)-, -N(benzyl)-, -N(alkyl C_1 to C_{20}-OH)-, -N(alkyl C_1 to C_{20}-NH_2)- and –N(alkenyl)-

The glycoside central unit is represented in the L- or D-configuration and the configuration at the anomeric position (1-position) of the glycoside central unit is alpha, beta or a mixture of both.

According to the invention the preferred glycoside units are monosaccharides like aldoses, preferred glucose; ketoses, preferred fructose; pyranoses, preferred mannose, glucose; furanoses, preferred ribose and arabinose. Examples of specific oligo- and polysaccharides, respectively consisting monosaccharide units, are lactose and melibiose. Typical monosaccharides are also amino sugars, preferred mannose amine, glucose amine and lactose amine.

Preferred acetal groups for **R** include $(CH_3O)_2$- and $(CH_3\text{-}CH_2O)_2$-, an aldehyde group is OHC-CH_2-O-, an alkenyl group is CH_2=CH-CH_2-O-, an acrylate group is CH_2=CH-CO_2-, a methacrylate group is CH_2=C(CH_3)-CO_2-, an acrylamide group include CH_2=CH-CONH-, an amino alkyl group is H_2N-CH_2-CH_2-, a protected amino alkyl group include W--NH-CH_2-CH_2--, wherein W is an amino protecting group, like Boc, Fmoc and Cbz, a thio alkyl group is HS-CH_2-CH_2--, and a protected thio alkyl group include V--S-CH_2-CH_2--, wherein V is a thiol protecting group.

The term "poly" used herein refers to a poly alkylene oxide like polyethylene glycols or polypropylene glycols, to a polyoxyalkylated polyol, like monomethoxy polyethylene glycols (mPEG) or oxyethylated triethanolamine, TEA(OE), to a poly olefinic alcohol, like polyvinyl alcohol.

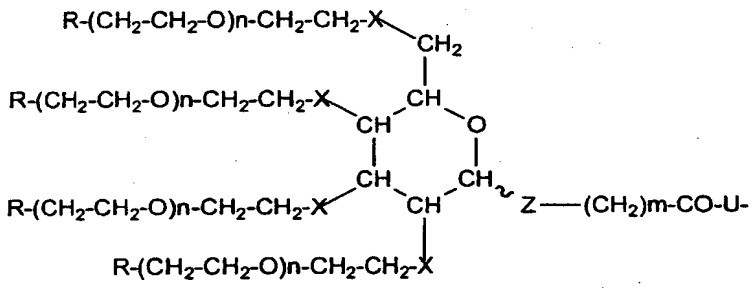
Polymers are preferred in which the polymer chain is terminated on one side by a methoxy group.

Furthermore, polymers are preferred in which the polymer chain is a polyethylene glycol, which is represented by the general structure:

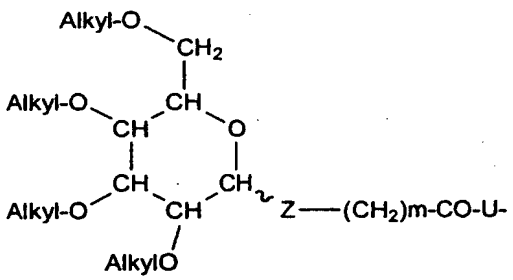
R--(CH_2-CH_2-O)n—CH_2-CH_2--X--

wherein
R and X are as defined above and n is 0 to 2000 or
wherein R is also a repeating building block, which has one of the following structures III or IV:

III



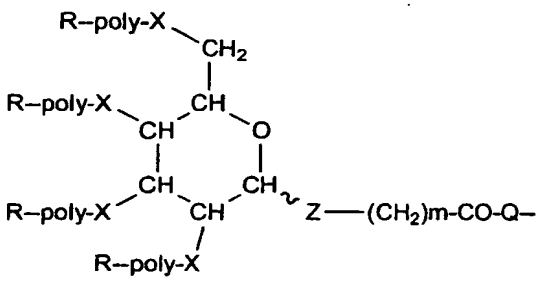
IV



wherein n, m, R, X and Z are as defined above,
alkyl means 1 to 20 C atoms and U is selected of -O-, -S- and -NH-,
whereas in the inventive protecting reagent the glycodendrimer repeating building block is commonly contained 4 to 1000 times.

Furthermore, the polymer chain can be an unsubsituted chain except for the terminus or can be also a random or block copolymer or a terpolymer.
Especially preferred are active protecting reagents with glucose as the glycoside central unit, which have the following structure V:

V



wherein m, poly, Q, R, X and Z are as defined above.

The active carbohydrate containing protecting reagents (I) of the present invention can be preferably prepared by an alkylation (Williamson Ethersynthesis) or other nucleophilic substitution reactions (e.g. urethane formation) between a glycoside, which contains a pentenyl group at the anomeric position, and a polymer. The reaction is carried out in solution. The pentenyl group of the resulting synthesis intermediate is converted to the active carbohydrate containing protecting reagent as shown in synthesis scheme 1 (Fraser-Reid, B.; Journal of the Organic Chemistry 2000, vol. *65*, pp. 958-963).

The preferred polymers used for the preparation of the active protecting reagents are polyethylene glycols, which are covalently attached to 4-penten-1-yl glycosides.
The pentenyl group is a multifunctional group, which can be converted to many different activated linkers. These polymer containing pentenyl glycosides are also used as building blocks in order to form larger, branched aggregates (glycodendrimers or parts of them). The branched, active carbohydrate containing protecting reagents react readily with amino functional groups of proteins and peptides in aqueous solution under mild reaction conditions. The dendritic compound is coupled to the polypeptide backbone by forming an amid, urethane, thiourethane, carbamates, ether, thioether, a secondary amine (reductive amination, reaction with a tresylated or halogenated linkage unit).

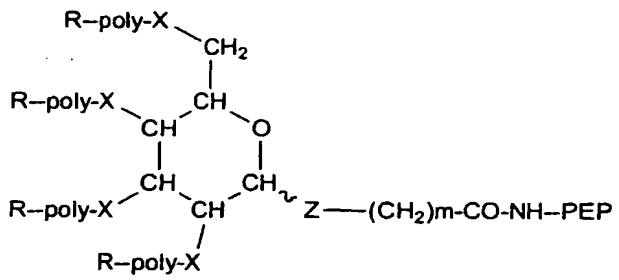
The active carbohydrate containing protecting reagents according to this invention does not have to be of a particular molecular weight. A preferred preparation of the active carbohydrate containing protecting reagent has a molecular weight between 500 and 60000, more preferably between 800 and 20000.

The preference of the active carbohydrate containing protecting reagents according to the present invention is first the fact that the small-sized reagents are mono-disperse compounds, which is an advantage in production of homogenous protecting reagents, analysis of conjugates with these reagents, in purification of these conjugates and in the definition of a specification in terms of regulatory issues.
The "umbrella-like" structured active carbohydrate containing reagents provided in this invention have proven protecting properties by the means of increased stability against proteolysis of the conjugated biologically active compound. The biological activity of such conjugates is maintained and during the modification reaction no cross-linking occurs. In contrast, unwanted cross linking reactions are major problems when common activated mPEG's are used for modification, because commercial available mPEG's contain relevant amounts of dihydroxy polyethylene glycol, which gets activated during the production process for mPEG's and then functions as cross linker.
In terms of the invention such branched reagents are more effective in protecting proteins from proteolysis and in reducing the immunogenicity.

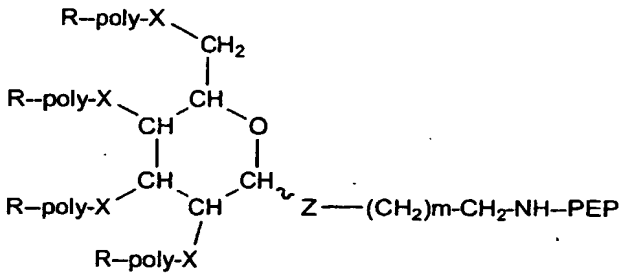
The active carbohydrate containing protecting reagents are used in the production of water soluble and isolatable conjugates with at least one biologically active molecule, a surface or a whole cell. The biologically active molecule is preferably selected from the groups of proteins, glycoproteins, peptides (e.g. human growth factors), enzymes, liposomes, antibodies, drugs, phospholipids, lipids, nucleosides, oligonucleotides, microorganisms, human cells, dyes and surfaces.

In a preferred manner the water soluble and isolatable conjugates have the structures VI or VII:

VI



VII



wherein m, poly, R, X and Z are as defined above and the unit NH-PEP represents an modified amino function (e.g. lysine group) of a biologically active molecule.

In terms of the present invention conjugates with the active carbohydrate containing protecting reagents are used to protect biologically active compounds, to increase the molecular weight of these biologically active compounds, thus reducing renal clearance, to prevent the formation of antibodies and antigen processing cells, respectively, and to minimize the proteolytic degradation. Furthermore, such a chemical modification is used to improve the bio-physical properties in terms of the solubility of drugs. The active carbohydrate containing protecting reagents are used to increase the solubility of hydrophobic active pharmaceutical ingredients (API's). These special kinds of pharmaceutical formulation facilitate the administration of hydrophobic drugs.

Furthermore, the active carbohydrate containing protecting reagents are also characterised by a good solubility in organic solvents, thus the modification of bio-catalysts (e.g. enzymes) with the new protecting reagents increase the solubility of the modified bio-catalysts in organic solvents, which is an interesting application for technical use.

Furthermore, the active carbohydrate containing protecting reagents according to the present invention are used to modify drug delivery and targeting systems (e.g. liposomes and antibodies), which opens new fields of application for these technologies.

Furthermore, the active carbohydrate containing protecting reagents are able to bind a biologically active molecule to other biologically active molecules, which may be the same or different, or to bind a biologically active molecule to a surface.

In a preferred manner the inventive reagents are suitable for the chemical modification of enzymes like asparaginase, glutaminase-asparaginase (PGA), glucocerebrosidase, of cytokines like alpha-Interferon, Interferon beta 1-b, G-CSF or of other therapeutically suitable proteins like TNF, human Insulin, antibodies or antibody fragments and small molecule drugs like doxorubicin and paclitaxel.

The new carbohydrate containing protecting reagents are particularly suitable for the chemical modification of pharmaceutical active ingredients. The pharmaceutical usefulness is characterized by a significantly increased protease stability (for example) of conjugates. Thus, the modification with the new carbohydrate containing protecting reagents will expand the circulation time *in-vivo*, which is required for pharmaceutical use of many biotechnological products.

The following examples are given to describe the invention, but should not limit the invention:

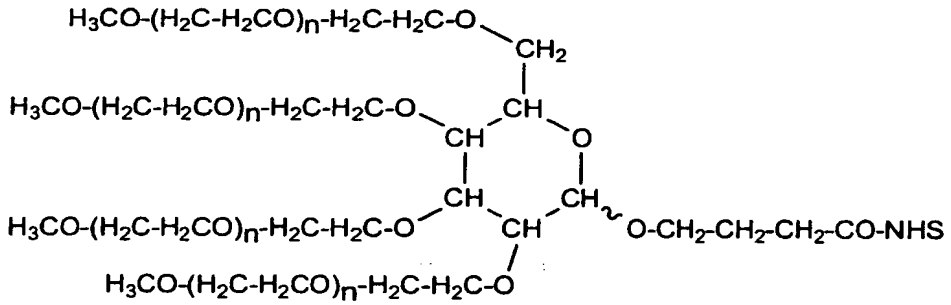
Example 1

Syntheses

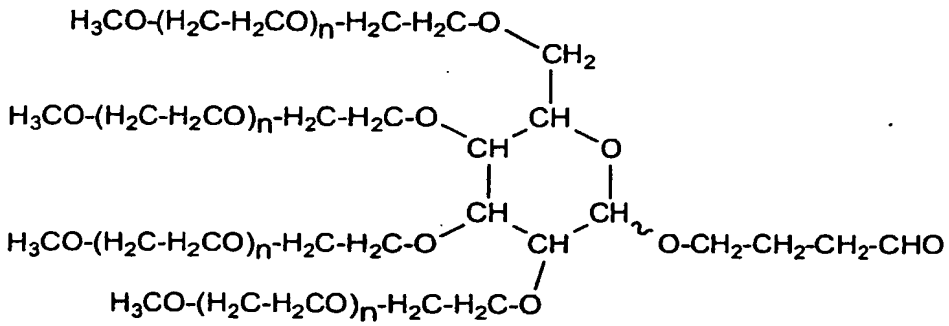
Example structures

In the schemes VIII and IX shown below the polyethylene glycol chains are covalently attached via an ether bond to the carbohydrate central unit.

VIII



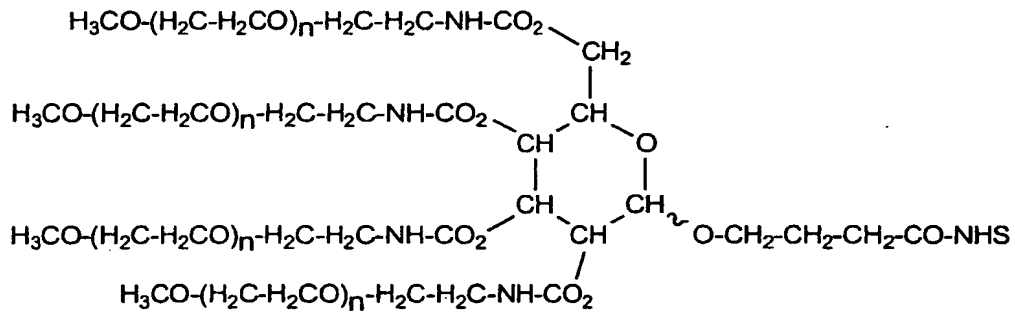
IX



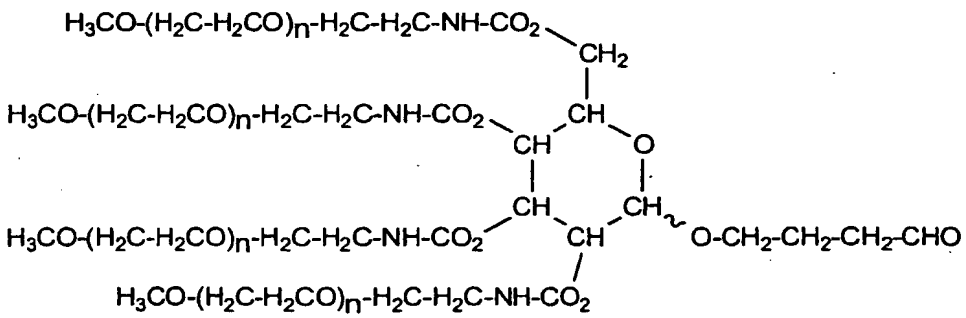
n is defined from 0 to 1000.

In the schemes X and XI shown below the polyethylene glycol chains are covalently attached via an carbamate bond to the carbohydrate central unit.

X



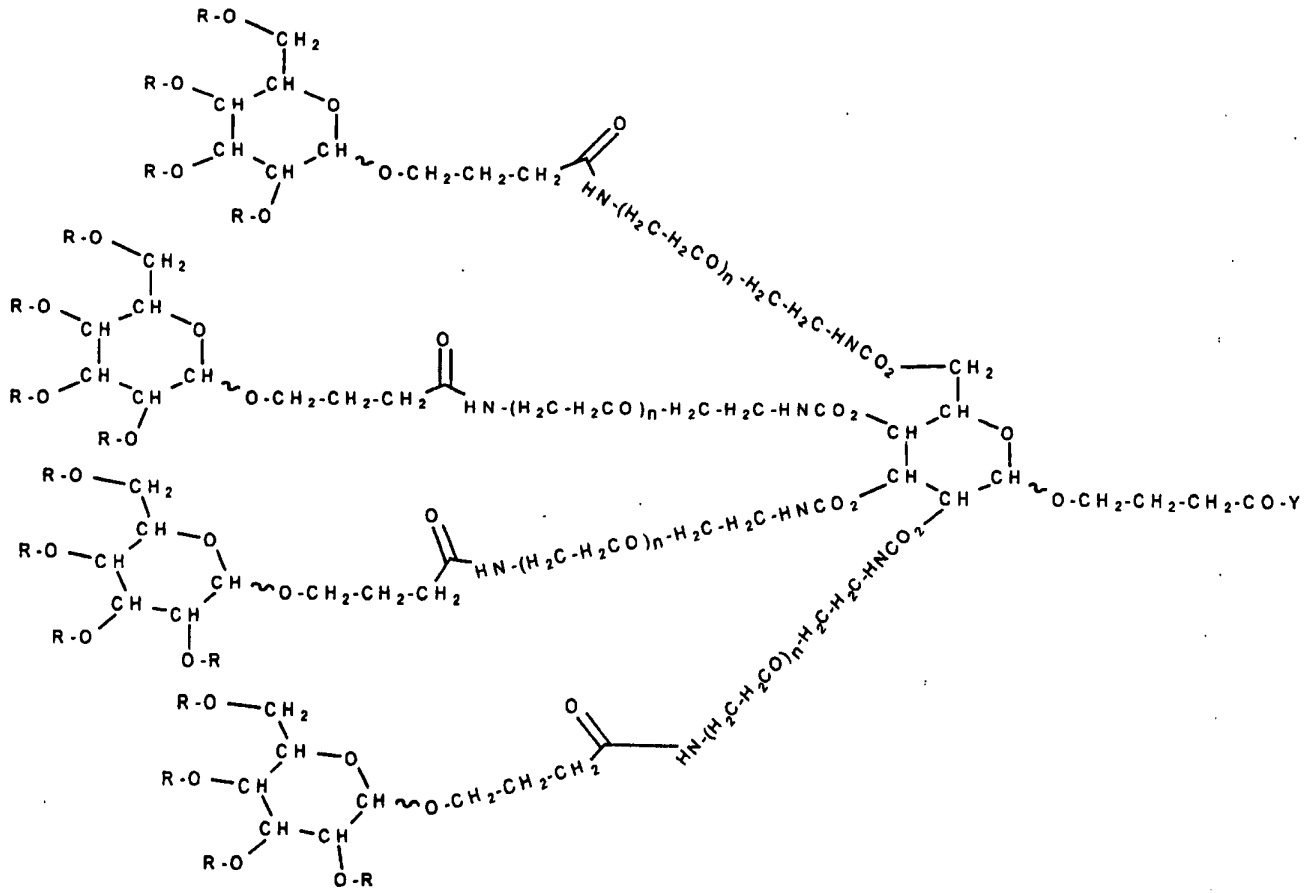
XI



n is defined from 0 to 1000.

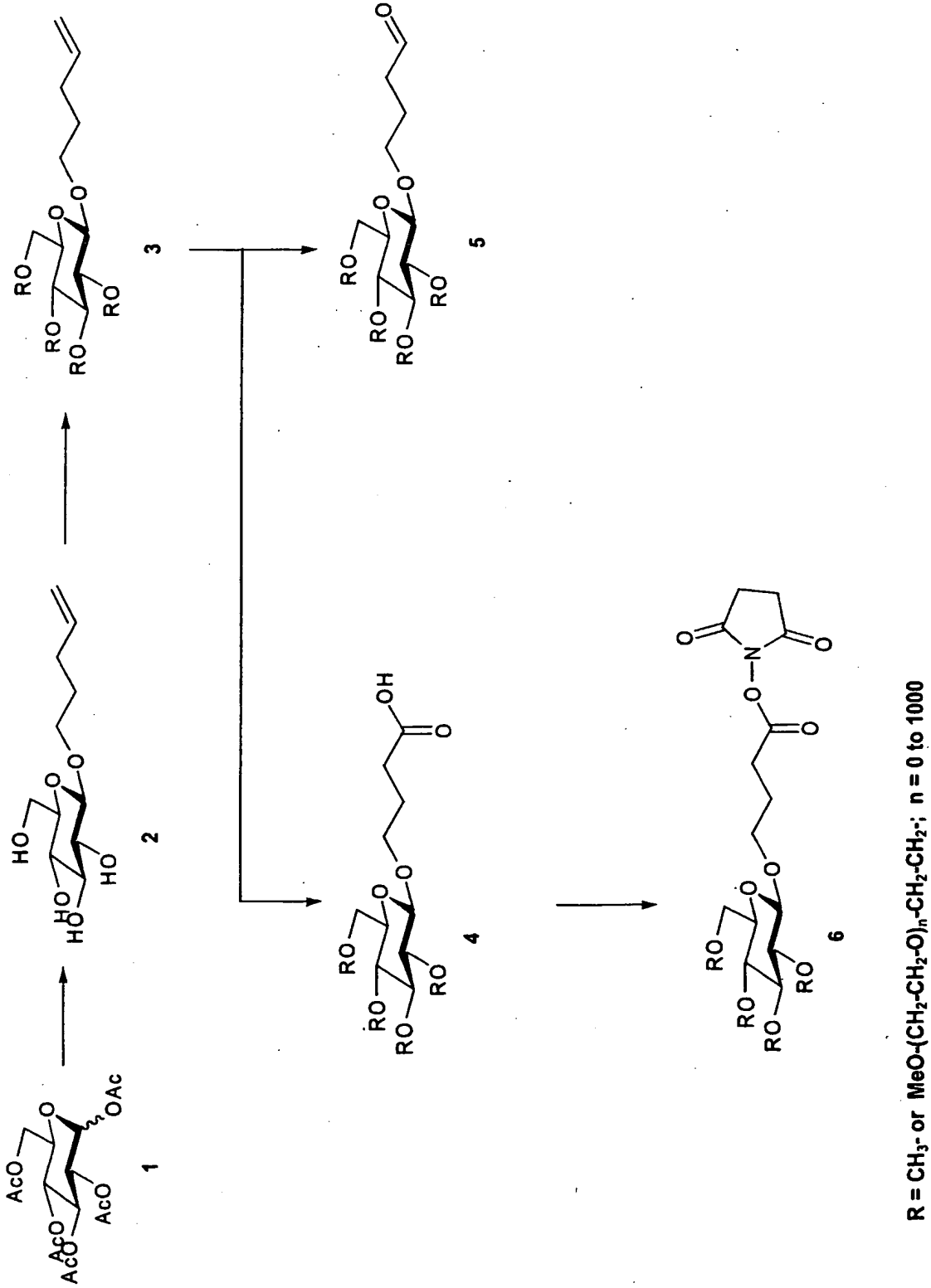
The following scheme XII shows branched, large sized structures connecting the two linkage strategies.

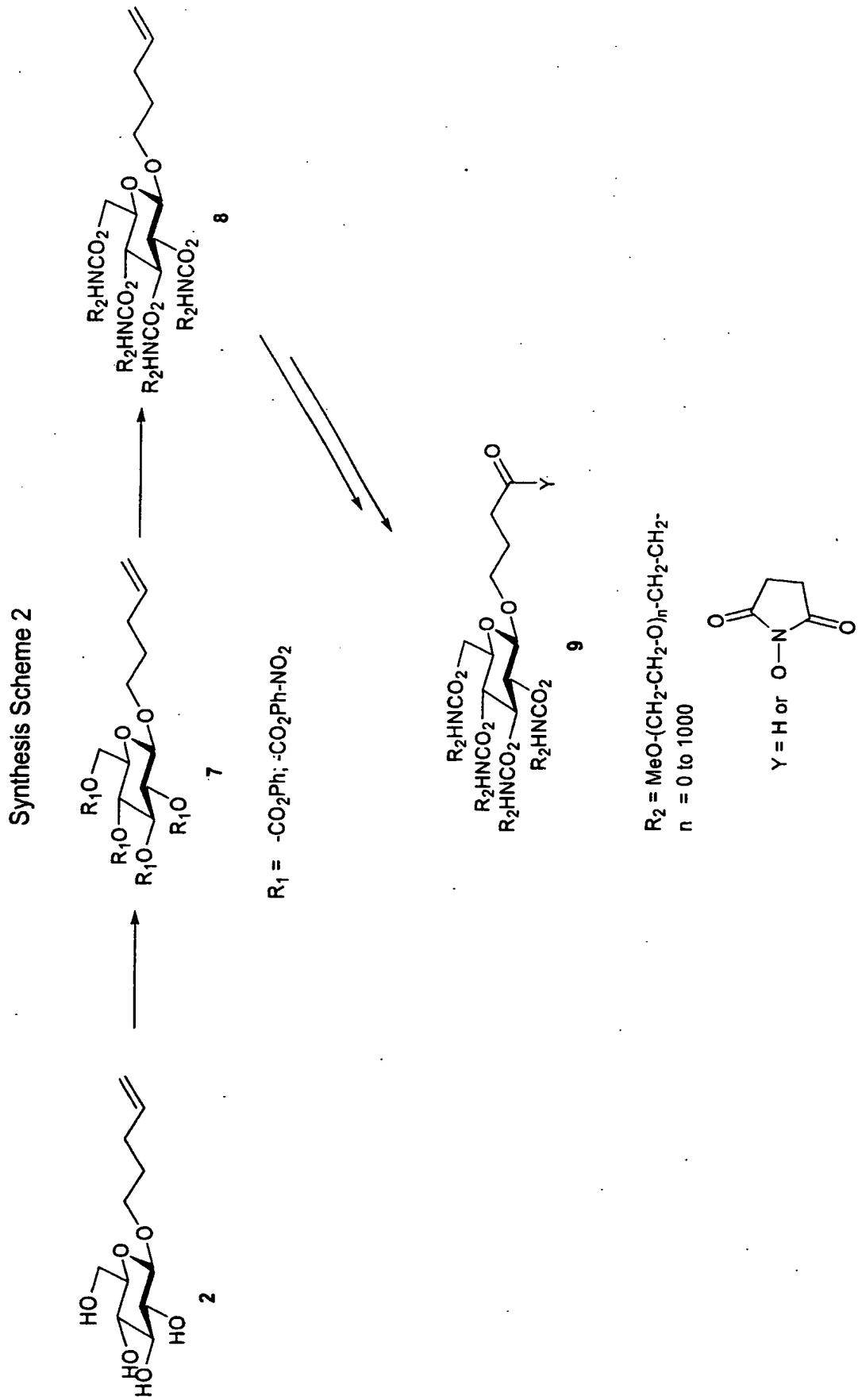
XII

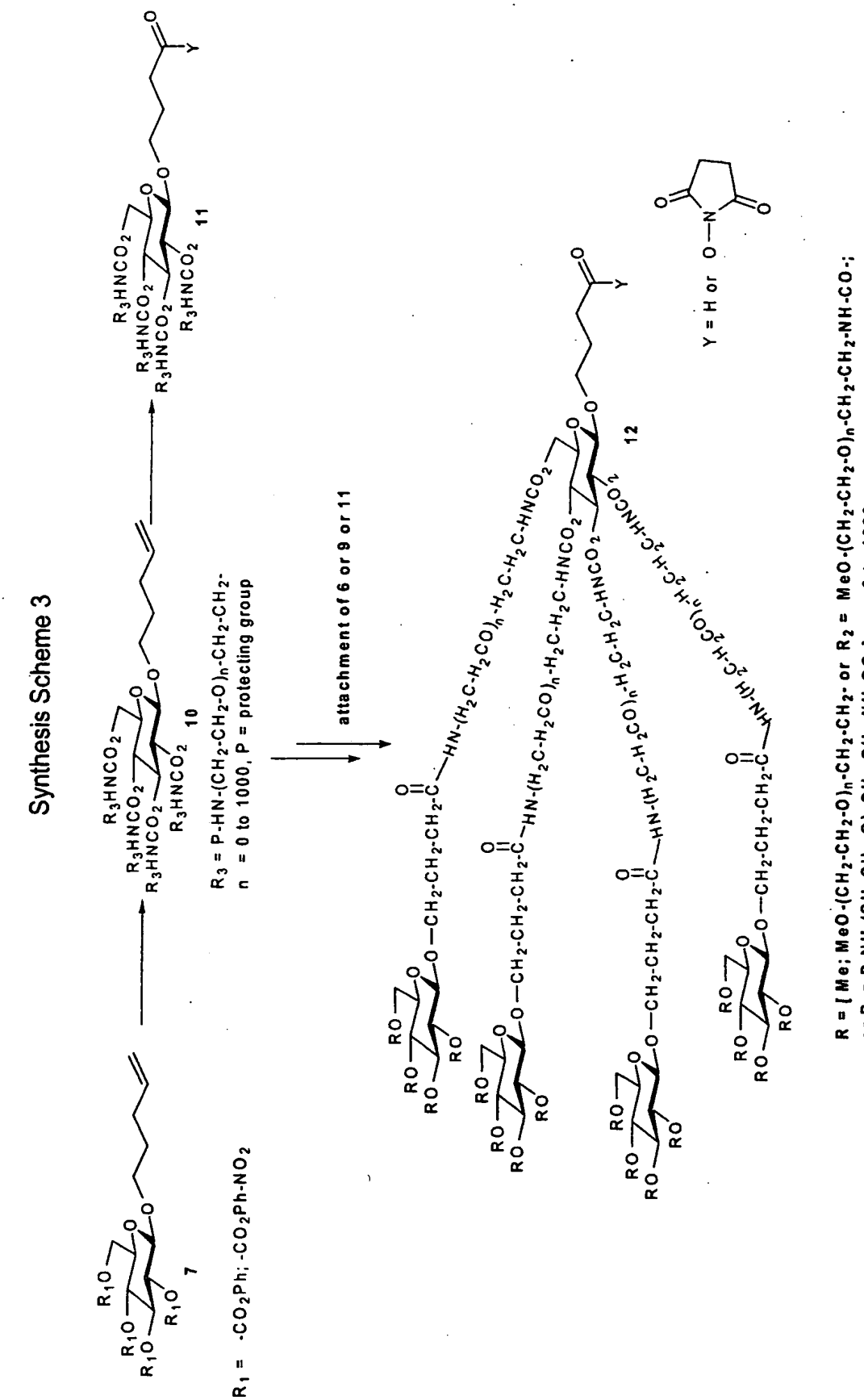


R = CH_3- or MeO-$(CH_2$-CH_2-O$)_n$-CH_2-CH_2-; Y = H or NHS, n = 0 to 1000

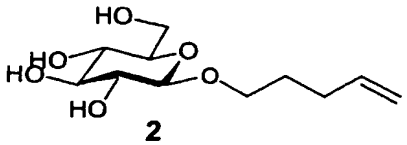
Synthesis Scheme 1







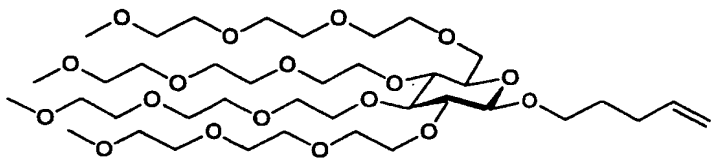
Example 1a
Synthesis of compound **6**



2

To a solution of 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranose (5 g) in dichloromethane (20 ml) and 4-penten-1-ol (2.7 mL) $BF_3 Et_2O$ (3.2 mL) is added at 20 – 30°C. The reaction mixture is stirred for 4 hrs at 20 – 25 °C. Afterwards the reaction mixture is diluted with dichloromethane (20 mL) followed by adding a NaOH solution (0.5 M, 70 mL), so that the pH of the reaction mixture is adjusted to about 6. The organic layer is separated and washed with a bicarbonate solution (5 %, 50 mL). Evaporation of the organic solvent gave the crude glycosylated product (6 g) as a yellow oil.

Sodium (320 mg) dissolved in methanol (4 mL) is added to a solution of the crude material (6 g) in methanol (26 mL) at 20 – 25 °C. Short after the addition of the sodium methylate a white precipitate is formed. The reaction mixture is stirred for 2 hrs at 20 – 25 °C. Afterwards the reaction is quenched by adding hydrochloric acid (1 M) until the pH of the reaction mixture is adjusted to 5 – 6. Evaporation of the solvent and purification of the crude material gave **2** (1.8 g) as a colourless oil. ^{1}H-NMR (250 MHz, $CDCl_3$): δ = 1.50 – 1.6 (m, 2H, CH_2-pentenyl), 2.05 -2.10 (m, CH_2-pentenyl), 3.20 - 4.00 (m), 4.10 - 4.6 (m), 4.70 – 5.10 (m, anomeric-H, CH_2=,), 5.60-5.75 (m, 1H, =CH-).
^{1}C-NMR (62.9 MHz, $CDCl_3$): δ = 28.52, 29.94, 61.20, 67.73, 69.54, 71.53, 71.96, 74.16, 102.84 (C-1), 115.04 , 137.95.



3

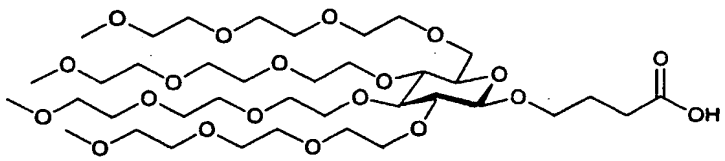
To **2** (2.2 g) dissolved in THF (50 mL) sodium hydride (1.5 g, 60 % in oil) is added at 20-25°C. The formed suspension is heated to 40 °C and stirred at this temperature for 30 min. After cooling to 20-25°C the mono-methoxytriethylene glycol p-toluensulfonate (13.3 g), which has been prepared before from the

corresponding alcohol, is added. The stirring is continued for 24 hrs at 20-25°C. Afterwards the reaction mixture is quenched by adding MeOH/water (5.0 mL). Extraction with ethyl acetate and dichloromethane, evaporating of the organic solvent and finally purification by flash chromatography gave **3** as colourless oil (1.66 g, 85 % yield).

^{1}H-NMR (250 MHz, $CDCl_3$): δ = 1.50 – 1.6 (m), 1.95-2.05 (m), 3.10-4.20 (m, contain all OCH_3, β-anomeric-H), 4.80-5.00 (m, CH_2=), 5.60-5.68 (m, 1H, =CH).

^{1}C-NMR (62.9 MHz, $CDCl_3$): δ = 29.10, 30.34, 59.18, 67.62, 68.00 - 74.00 (signals overlapped), 78.28, 82.30, 83.00, 85.14, 103.39 (β–C-1), 115.02, 138.30.

ESI-MS m/z: 833.5 $[M+H]^+$, 855.5 $[M+Na]^+$ (positive +ESI Mode, Finnigan AQA)



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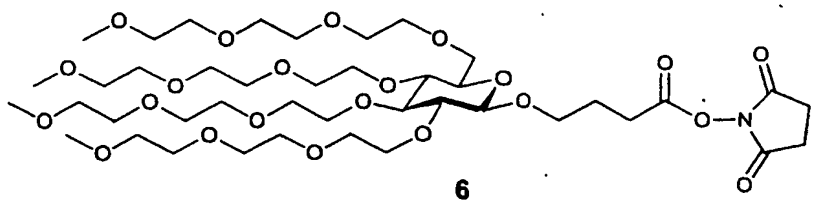
To a solution of $NaIO_4$ (1.91 g) and $RuCl_3$*H_2O (17.8 mg) in a mixture of dichloromethane/acetonitrile/water (2:2:3, 40 mL) **3** (1.8 g) is added at 20 – 25 °C. While stirring 200 µl glacial acetic acid is added. After stirring of the reaction mixture for 2 hrs a second amount of $NaIO_4$ (1.9 g) is added. Further stirring for 2 hrs at 20 – 25°C is driving the reaction to completion. Afterwards, the reaction mixture is diluted with water and extraction with dichloromethane (50 mL) gave the crude product. Purification via flash chromatography gave **4** as a light yellow oil (1.82 g, 97 % yield). ^{1}H-NMR (250 MHz, $CDCl_3$): δ = 1.75 -1.90 (m, CH_2), 2.35 - 2.45 (m, CH_2), 3.10 - 4.00 (m, contain all OCH_3, OCH_2, OCH), 4.15 (d, 1-Hβ, J = 8 Hz).

^{1}C-NMR (62.9 MHz, $CDCl_3$): δ = 24.67, 30.25, 52.68, 58.75 – 78.61 (signals overlapped), 82.45, 84.80, 102.73 (beta C-1), 175.95.



6

To a solution of **4** (1.8 g) in dichloromethane (10 mL) DCC (686 mg) and NHS (308 mg) is added. The reaction mixture is stirred for 15 hrs at 20 – 25°C. Afterwards the precipitated urea is filtered and the filtrate is evaporated. Purification by flash chromatography gave the active ester **6** as light yellow viscous oil (1.033 g, 51 % yield). ^{1}H-NMR (250 MHz, $CDCl_3$): δ = 1.75 -1.90 (m, CH_2), 2.50 -2.65 (m, CH_2), 2.70 (s, 2 x CH_2), 3.15 - 4.00 (m, contain OCH_3, OCH_2, OCH), 4.15 (d, 1-Hβ, J = 8Hz). ^{1}C-NMR (62.9 MHz, $CDCl_3$): δ = 24.67, 25.28 (2 x CH_2, NHS), 27.48, 58.67, 66.00 - 72.20 (signals overlapped), 74.57, 77.75, 82.34, 84.69, 102.88 (C-1), 168.20, 168.82. ESI-MS m/z: 948 $[M+H]^+$, 970 $[M+Na]^+$ (positive +ESI Modus, Finnigan AQA).

Example 1b:

Synthesis of compound **7** with R_1 = -CO_2-Ph

2 (2.0 g, synthesised as described in Example 1a) is dissolved in a mixture of THF (25 mL) DBU (1 mL) and pyridine (7 mL). The reaction mixture is cooled to 0 °C. While stirring phenyl chloroformate (4.3 mL) is added dropwise over a period of 30 min (exothermic reaction). Afterwards the reaction mixture is stirred for 24 hrs at 20 – 25 °C. The reaction mixture is diluted with ethyl acetate (50 mL) followed by adding sodium bicarbonate solution (5 %, 35 mL). Phase separation, solvent evaporation and purification by flash chromatography gave **7** (6.1 g, product contains some phenol) as viscous foam. ^{1}H-NMR (250 MHz, $CDCl_3$): δ = 1.65 - 1.80 (m, CH_2), 2.00 - 2.20 (m, CH_2), 3.45 - 3.55 (m), 3.90 – 4.10 (m), 4.45 – 4.55 (m), 4.6 – 4.70 (m), 4.85 – 5.45 (m), 5.60 – 5.75 (m, =CH-), 7.00 – 7.50 (m, phenyl protons, 20 H). ^{1}C-NMR (62.9 MHz, $CDCl_3$): δ = 28.58, 29.82 (2 x CH_2), 65.86, 69.58, 71.02, 73.03, 75.62, 77.23, 100.39 (beta C-1), 115.19 (CH_2=),120.82, 120.95, 121.01, 126.12, 126.22, 126.30, 129.42, 137.63 (=CH-), 150.85, 150.93, 150.97, 152.52, 152.77, 153.19, 153.51.

Example 2
Modification of biological active compounds

The following examples are given to describe the usefulness of the present invention, but should not limit the invention:

General methods: Protein concentration is determined by the Lowry method (Lowry et al., 1951). The electrophoresis experiments (SDS-Page) are carried out as described by Laemmli (1970) using 12.5 % polyacrylamid gels and stained with Coomassie Brilliant Blue R-250. The enzymes L-Asparaginase, e.g. from Escherichia coli, and Pseudomonas 7A Glutaminase-Asparaginase (PGA) are amido hydrolyses catalyzing the deamidation of the amino acids L-Asparagine and L-Glutamine, respectively. During the reaction ammonia is released, which can be determined by using Nessler's reagent as described by Roberts, J. (1976). The working pH for L-Asparaginase is 8.6 and for PGA 7.2.

Reference Proteins: An L-Asparaginase modified with polyethylene glycol (PEG-Asparaginase) was purchased from Sigma. An Pseudomonas 7A Glutaminase-Asparaginase modified with polyethylene glycol (PEG-PGA) was provided by Medical Enzymes AG. Each PEG chain used for modifying these enzymes has a molecular weight of 5000 g/mol.

Example 2a
Preparation of modified L-Asparaginase (short "ASNase"):

The reactions described below were carried out in Eppendorf test tubes. To a solution of L-Asparaginase (ProThera GmbH, 10.4 mg/mL) in bicarbonate buffer (0.05 M, pH 8.5 – 9) compound **6** (14.4 µL, 0.8 eq.) dissolved in DMSO (200 mg/mL) is added. The reaction mixture is slightly stirred at 20 – 25 °C for 30 min. The unreacted and hydrolyzed protecting reagent (**6**) is removed by diafiltration using water. The extent of modification of L-Asparaginase is 26 % as determined by ***Matrix Assisted Laser Desorption Ionization – Mass Spectrometry*** (MALDI-MS; matrix: sinapinic acid). Alternatively, the extent of modification can be determined by trinitrobenzenesulfonate titration of the amino functional groups as described by Habeeb (Anal. Biochem. **1966**, *14*, 328-336).

In a second experiment L-Asparaginase is modified using a larger excess of the protecting reagent **6** (32.3 µL, 1.8 eq.). The reaction and the purification is performed as described above. The modification gave an extent of modification of 35 % determined by MALDI-MS.
Each of the modified L-Asparaginase samples preserved 95 % of the enzymatic activity in comparison with the unmodified L-Asparaginase.
Fig. 1 shows the analysis of different L-Asparaginase preparations by SDS-PAGE.

Example 2b
Preparation of modified Pseudomonas 7A Glutaminase-Asparaginase (PGA):

These modification reactions were carried out under the same conditions and with the same materials as described for L-Asparaginase (example 2a). The modification reaction of PGA using 0.8 eq. of the protecting reagent **6** resulted in an extent of modification of 32 % as determined by MALDI-MS. In the case of modified PGA 90 % of the enzymatic activity is preserved in comparison with the unmodified PGA.

The second preparation is produced by using 1.8 eq. of **6**, which gave an extent of modification of 41 % as determined by MALDI-MS. The enzymatic activity of modified PGA was reduced to 85 % of the activity without such a modification.
Fig. 2 shows the analysis of different PGA preparations by SDS-PAGE.

Example 3
***In-vitro* assay to proof the usefulness of the present invention**

As a degree for the usefulness of the new carbohydrate containing protecting reagents in pharmaceutical applications, the stability of modified L-Asparaginase and modified PGA, respectively, against the protease Trypsin was investigated in comparison with the unmodified enzymes. Modified L-Asparaginase and modified PGA are prepared as described in example 2a and example 2b, respectively. In addition, an enzyme fraction named "blank" is treated in the same manner as described in examples 2a and 2b, but without addition of active carbohydrate containing protecting reagent. Test tubes contained 1–2 IU/mL of the "blank", modified and unmodified enzyme, respectively, and are incubated at 37°C in Tris/Cl buffer solution in the presence of 0.25µg/mL Trypsin. The control is

unmodified enzyme from the mother enzyme solution incubated at 37°C in Tris/Cl buffer solution in the absence of Trypsin. After 0, 10, 30, 60 and 90 min the enzyme activity is determined from an aliquot of each test tube and the control.

As it is showed in Fig. 3 and Fig. 4 the incubation of the enzymes L-Asparaginase and PGA, respectively, in the presence of Trypsin results in significant loss of enzyme activity after 30 to 90 min. In the case of L-Asparaginase, this reduction in enzyme activity can be completely, and in the case of PGA partly avoided by modifying the enzymes with active carbohydrate containing protecting reagents as described in the present invention. In conclusion, the modification of the enzymes L-Asparaginase and PGA leads to a significant increase of stability against degradation by the protease Trypsin.

Legends to the figures:

Fig. 1:

Analysis of different L-Asparaginase preparations by SDS-PAGE. Proteins are stained with Coomassie. Modifications are performed with L-Asparaginase provided by ProThera. The samples are: Lane 1) L-Asparaginase (ProThera 2,5 µg), Lane 2) PEG-L-Asparaginase (Sigma, 5 µg), Lane 3) recombinant protein standard (Amersham Bioscience, RPN 800), Lane 4) modified L-Asparaginase (0.8 eq. 6, 2.5 µg), and Lane 5) modified L-Asparaginase (1.8 eq. of 6, 2.5 µg).

Fig. 2:

Analysis of different PGA preparations by SDS-PAGE. Proteins are stained with Coomassie. Modifications are performed with L-Asparaginase provided by ProThera. The samples are: Lane 1) PGA Medical Enzymes 2,5 µg, Lane 2) PEG-PGA Medical Enzymes, 5 µg, Lane 3) recombinant protein standard (Amersham Bioscience, RPN 800), Lane 4) Modified PGA (0.8 eq. 6, 2.5 µg), and Lane 5) Modified PGA (1.8 eq. 6, 2.5 µg).

Fig. 3:

Influence of the modification of L-Asparaginase with active carbohydrate containing protecting reagent on stability against degradation by Trypsin as derived from the remaining enzyme activity

Fig. 4:

Influence of the modification of Pseudomonas 7A Glutaminase-Asparaginase with active carbohydrate containing protecting reagent on stability against degradation by Trypsin as derived from the remaining enzyme activity

Claims:

1.) An active carbohydrate containing protecting reagent represented by a carbohydrate central unit G which is attached

a. to a polymer chain at least at one of the hydroxyl groups and

b. to a linkage unit at the anomeric position (1-position)

and which is represented by the general formula I

R-poly-X-G
ζ
Z - (CH_2)m – Y **I,**

wherein

G is a glycoside central unit, which is a monosaccharide, an oligo- and polysaccharide, respectively consisting monosaccharide units, an amino carbohydrate or a polyol,

R-poly- is

a polymer chain with one ore more recurring monomer units, or

a repeating building block **II** which is attached via the linkage group to a further glycoside unit G

P-HN-poly-X-G
ζ
Z - (CH_2)m – Y **II,**

wherein P is an amino protecting group or the next repeating building block or a terminal unit **I**

in which

R is H, OH or selected from the group consisting of alkyl, O-alkyl (alkyl is containing1 to 20 C atoms), benzyl, aryl, acetal, aldehyde, alkenyl, acrylate, acrylamide, active sulfone, alkyl amine, protected alkyl amine, thiol and protected thiol, or also selected from the group consisting of a monosaccharide, an oligo- and polysaccharide, respectively consisting monosaccharide units, an amino carbohydrate or a polyol, and

poly is selected from a poly alkylene oxide, a poly oxyethylated polyol, a poly olefinic alcohol and a poly acrylomorpholine

X is selected from --O--, --S--, --NH--, -NHCO--, --CONH-, -$NHCO_2$--, O_2CNH--, -CSNH-- and --NHCS--;

m is 1 to 10

Y is selected from a group of $(O\text{-alkyl})_2$, $-OSO_2CH_2CF_3$ (Tresyl), – CO – Q, maleimide, -O-CO-nitrophenyl or – trichlorophenyl, -S-alkyl (C_1 to C_6), -S-S-alkyl, -S-aryl, -S-S-aryl, -S-benzyl, -S-alkyl (C_1 to C_6)-OH, -S-alkyl(C_1 to C_6)-NH_2, -S-alkyl(C_1 to C_6)-$OSO_2CH_2CF_3$ and -S-alkyl(C_1 to C_6)-CO_2-Q-, -SO-alkenyl, -Halogen (Cl, Br, I), wherein

Q is H or OH or is selected from a group of O-aryl, O-benzyl, O-N-succinimide, O-N-sulfosuccinimide, O-N-phthalimide, O-N-glutarimide, O-N-tetrahydrophtalimide, -N-norbornene-2,3-dicarboximide, hydroxybenzotriazole and hydroxy-7-azabenzotriazole

and

Z is selected from -O-, -S- or –NH-, -N(alkyl C_1 to C_{20})- , -N(aryl)-, -N(benzyl)-, -N(alkyl C_1 to C_{20}-OH)-, -N(alkyl C_1 to C_{20}-NH_2)-.

2.) The reagent according to claim 1, wherein the carbohydrate central unit is represented in the L- or D-configuration.

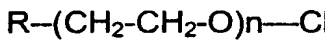
3.) The reagent according to any one of claims 1 to 2, wherein the configuration at the anomeric position (1-position) of the glycoside central unit is alpha, beta or a mixture of both.

4.) The reagent according to any one of claims 1 to 3, wherein the monosaccharide is an aldose, preferred glucose; a ketose, preferred fructose; a pyranose, preferred mannose, glucose; a furanose, preferred

ribose, arabinose; an oligo- and polysaccharide, respectively consisting monosaccharide units, preferred lactose, melibiose and an amino carbohydrates and amino carbohydrate containing oligo- or polysaccharides, respectively, preferred mannose amine, glucose amine, lactose amine.

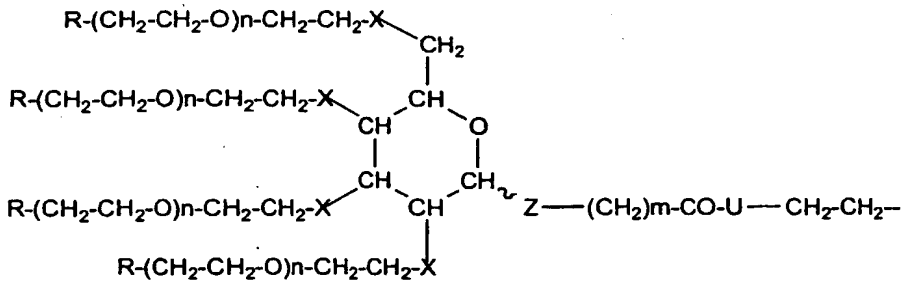
5.) The reagent according to any one of claims 1 to 4, wherein the polymer chain is terminated by a methoxy group.

6.) The reagent according to any one of the claims 1 to 5, wherein the polymer chain is a polyethylene glycol, which is represented by the general structure:



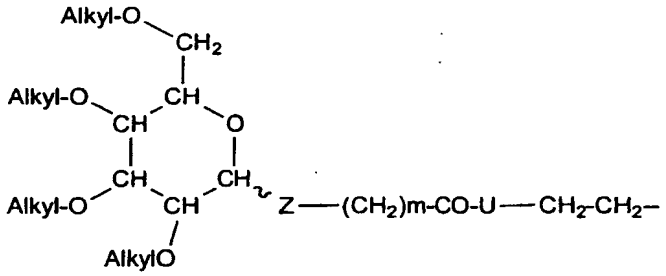
wherein
R and X have one of the above-mentioned meaning and n is 0 to 2000.

7.) The reagent according to claim 6, wherein R is a rest of formula II or a repeating building block, which has the following structure:



wherein n, m, R, X and Z have the above-mentioned meaning,
U is selected of --O--, --S-- and --NH--.

8.) The reagent according to claim 6, wherein R is a repeating building block, which has the following structure:



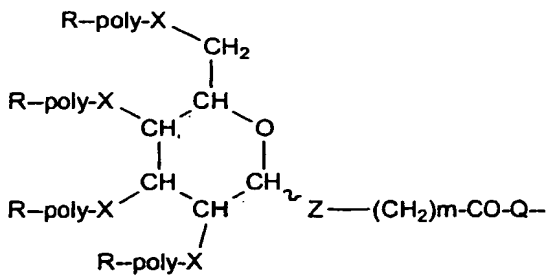
wherein n, m, R, X and Z have the above-mentioned meaning,
alkyl is an alkyl group with 1 to 20 C atoms and
U is selected of --O--, --S-- and --NH--.

9.) The reagent according to claim 7 or 8, wherein the building block repeats 4 to 1000 times.

10.) The reagent according to any one of claims 1 to 9, wherein the polymer chain is an unsubstituted chain except for the terminus.

11.) The reagent according to any one of claims 1 to 9, wherein the polymer chain is a random or block copolymer or a terpolymer.

12.) The reagent according to any one of claims 1 to 11, which has the following structure:



wherein m, poly, Q, R, X and Z have one of the above-mentioned meaning.

13.) A method for preparing the active carbohydrate containing protecting reagent of any one of claims 1 to 12, wherein the conjugation reaction between a glycoside, which contains a pentenyl group at the anomeric position of the glycoside, and an activated polymer chain, is carried out in solution, followed by conversion of the pentenyl group of the desired synthesis intermediate into the activated carbohydrate containing protecting reagent as shown in formula I and a method for purification of the activated reagent from the reaction mixture.

14.) Use of an active carbohydrate containing protecting reagent according to any one of claims 1 to 12 in the manufacture of a water soluble and isolatable conjugate with at least one biologically active molecule, a surface or a whole cell.

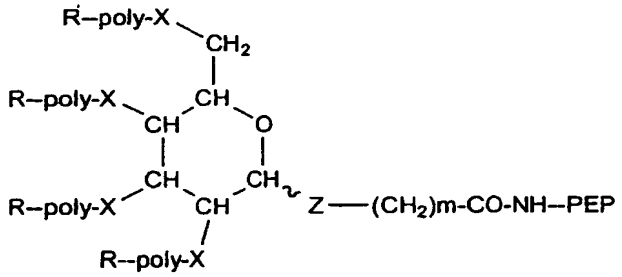
15.) The use according to claim 14, wherein the at least one biologically active molecule is selected from the group consisting of proteins, enzymes, glycoproteins, polypeptides, drugs, dyes, nucleosides, oligonucleotides, antibodies, lipids, liposomes, phospholipids, liposomes, microorganisms, human cells and surfaces.

16.) The use according to claim 15, wherein the at least one biologically active molecule is an enzyme.

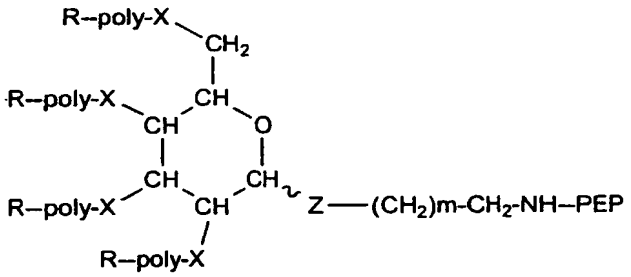
17.) The use according to claim 16, wherein the enzyme is asparaginase or glutaminase-asparaginase.

18.) The use according to claim 14, wherein the compound serves to bind a biologically active molecule to an other biologically active molecule, which may be the same or different, or to bind a biologically active molecule to a surface.

19.) The use according to claim 14, that the water soluble and isolatable conjugates having the structure:



or



wherein m, poly, R, X and Z have the above-mentioned meaning and the unit NH-PEP represents an amino function containing residue on a biologically active molecule.

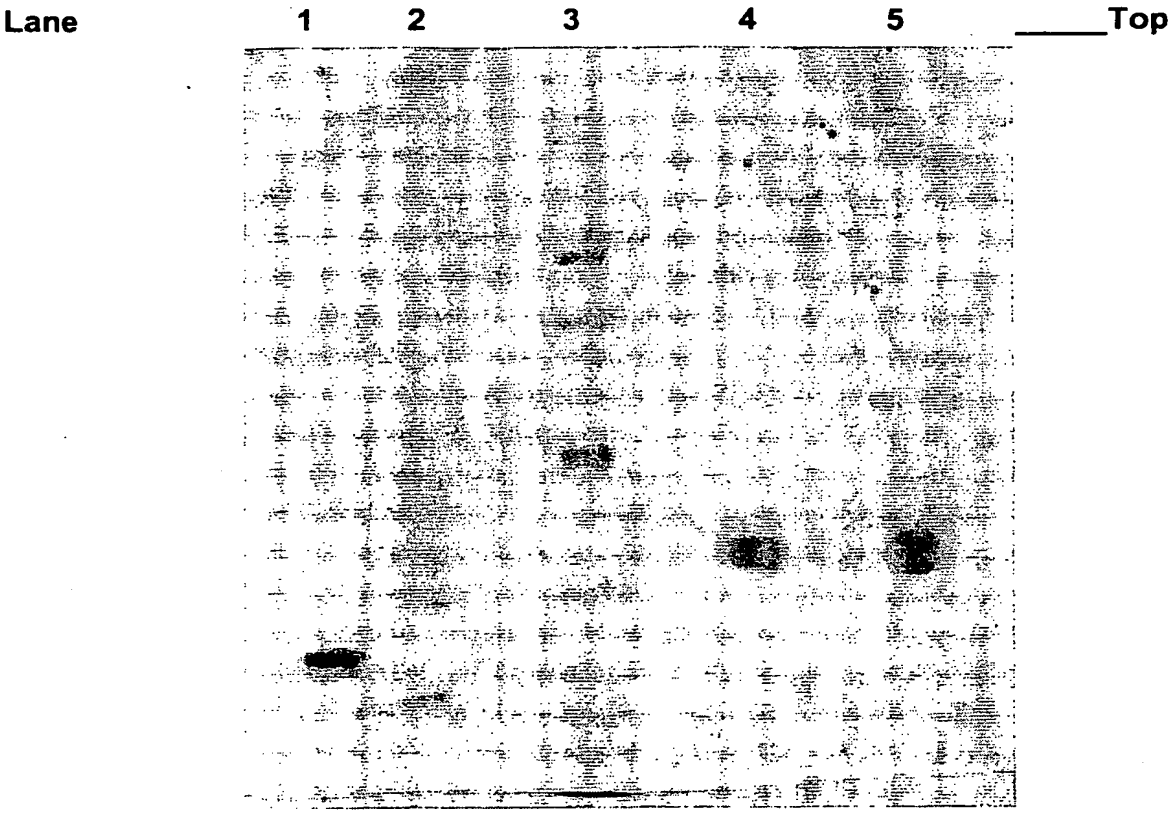


Fig. 1: *SDS-Page of the L-Asparaginase preparations*

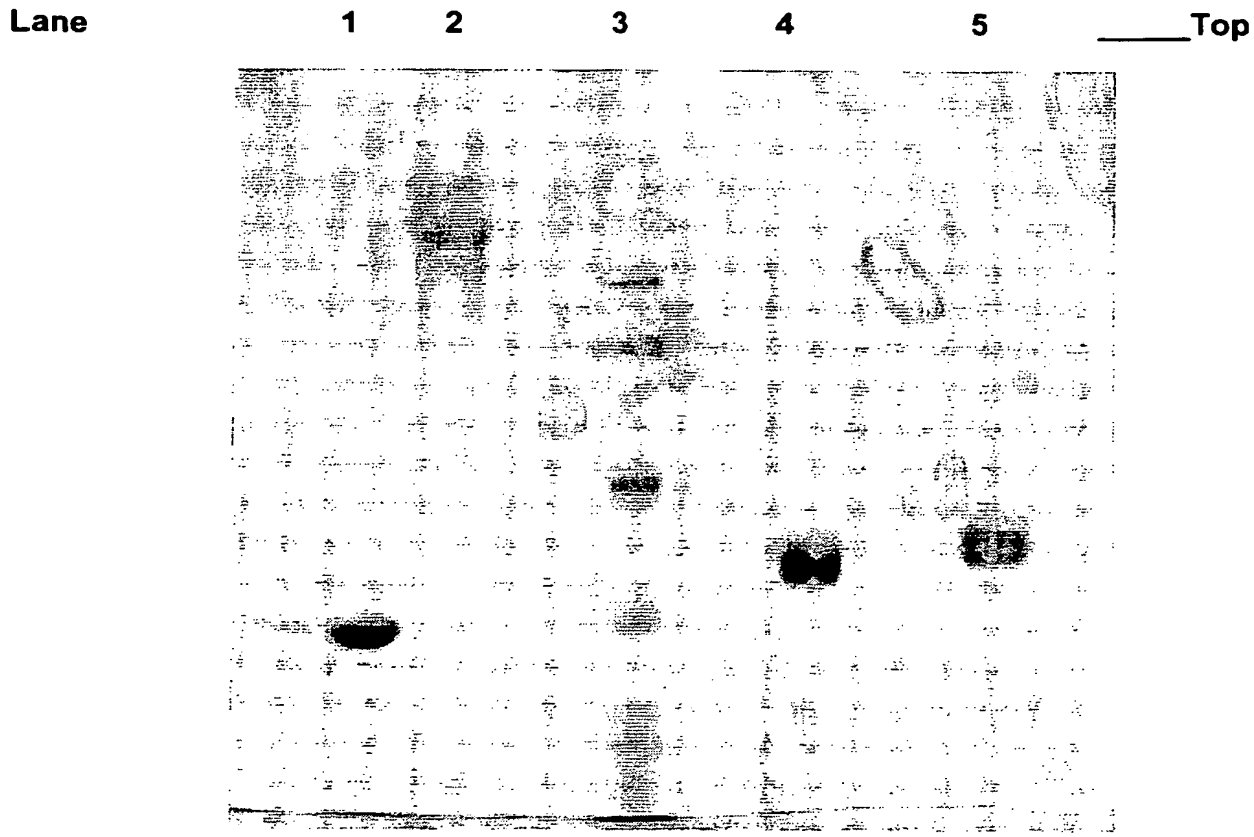


Fig. 2: *SDS-Page of the PGA preparations*

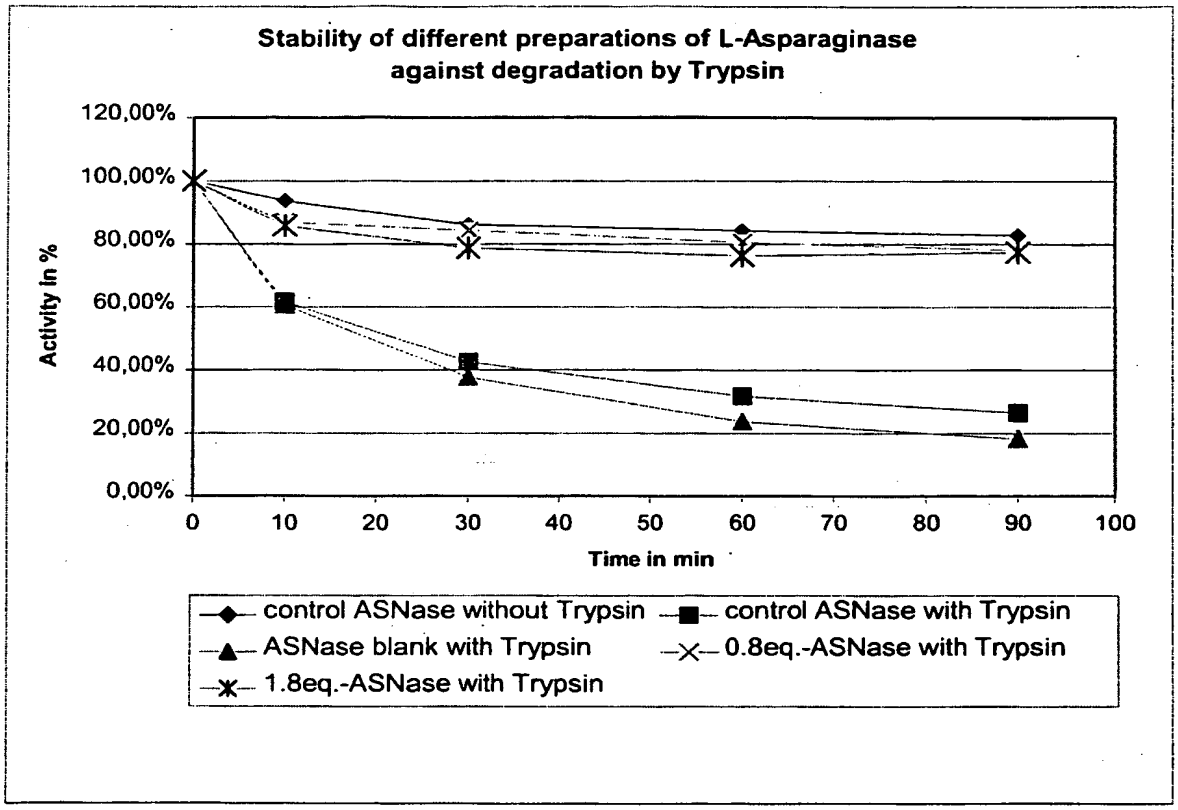

Stability of different preparations of L-Asparaginase against degradation by Trypsin
120,00%
100,00%
80,00%
60,00%
40,00%
20,00%
0,00%
Activity in %
0
10
20
30
40
50
60
70
80
90
100
Time in min
control ASNase without Trypsin
control ASNase with Trypsin
ASNase blank with Trypsin
0.8eq.-ASNase with Trypsin
1.8eq.-ASNase with Trypsin

Fig. 3

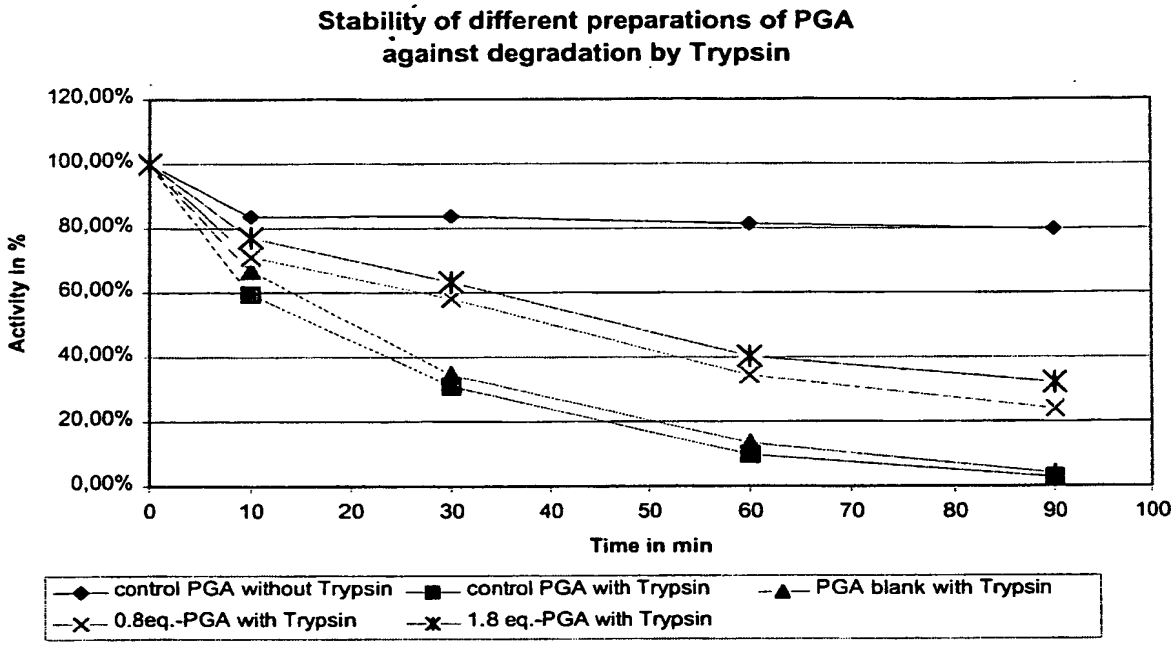

Stability of different preparations of PGA against degradation by Trypsin
120,00%
100,00%
80,00%
60,00%
40,00%
20,00%
0,00%
Activity in %
0
10
20
30
40
50
60
70
80
90
100
Time in min
control PGA without Trypsin
control PGA with Trypsin
PGA blank with Trypsin
0.8eq.-PGA with Trypsin
1.8 eq.-PGA with Trypsin

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No
PC 03/04790

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C08B37/00 C08G83/00 C08G65/331 C07K2/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C08G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
COMPENDEX, WPI Data, PAJ, CHEM ABS Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI MINGIN ET AL.: "The chemical modification of E. coli L-asparaginase with O-carboxymethylated chitosan" CHINESE JOURNAL OF REACTIVE POLYMERS, vol. 7, no. 1, 1998, pages 49-53, XP008021765 China the whole document ---	1,14-17
A	WILEMAN THOMAS E. ET AL.: "Soluble asparaginase-dextran conjugates show increasede circulatory persistence and lowered antigen reactivity" JOURNAL OF PHARMACY AND PHARMACOLOGY, vol. 38, no. 4, 1986, pages 264-271, XP008021760 UK the whole document ---	1,14-17

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 October 2003	22 OCT 2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mazet, J-F

orm PCT/ISA/210 (second sheet) (July 1992)

International Application No
PC . › 03/04790

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 99 01479 A (NATIONAL STARCH AND CHEMICAL INVESTMENT HOLDING CORPORATION) 14 January 1999 (1999-01-14) claims	1-6,14
A	ZALIPSKY S: "CHEMISTRY OF POLYETHYLENE GLYCOL CONJUGATES WITH BIOLOGICALLY ACTIVE MOLECULES" ADVANCED DRUG DELIVERY REVIEWS, AMSTERDAM, NL, vol. 16, no. 2/3, 1995, pages 157-182, XP002037428 ISSN: 0169-409X page 176, right-hand column, line 1 - line 28	1-6,10, 13-19
A	WO 97 48711 A (DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS) 24 December 1997 (1997-12-24) page 2, line 16 - line 29 page 2, line 30 -page 4, line 22 page 5, line 10 - line 27 claims; figures	1-4,9, 13-19
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A	EP 0 852 243 A (KATAOKA KAZUNORI) 8 July 1998 (1998-07-08) abstract	1

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